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Date: February 19, 2002 By: Kay L. Gaviglio

PATENT
Docket No. GC561-3C1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	
H. Wang et al.,	)	Group Art Unit: Unassigned
	)	
Serial No. Cont of 09/468,578	)	Examiner: Unassigned
	)	
Filed: Herewith	)	
	)	
For: PHENOL OXIDIZING ENZYMES	)	

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Sir:

Please enter the instant amendment, prior to considering the above-identified application on the merits.

In The Specification.

Please delete the Abstract at page 35 and replace it with the following:

ABSTRACT

The invention relates to phenol oxidizing enzymes encoded by nucleic acids capable of hybridizing to the nucleic acid of SEQ ID NO: 1, and fragments thereof. Particularly provided are nucleic acid sequences and amino acid sequences from *Bipolaris spicifera, Curvularia pallescens* and *Amerosporium atrum*. The invention also provides

expression vectors and host cells comprising nucleic acids encoding the phenol oxidizing enzymes, methods of producing said enzymes and methods for constructing host cells producing said enzymes.

Please also amend the following sections of the specification. A marked-up version of each amended section is provided as Appendix I herein.

A. Starting at the top of page 21 through page 22 of Example I, please amend the specification as follows:

- - The phenol oxidizing enzyme activity of the suspension was then measured using the standard assay procedure, based on the oxidation of ABTS by oxygen, as was described above (but with the exception that the preparation being assayed is the resuspended concentration and not the supernatant dilutions). The phenol oxidizing enzyme activity so measured was 5200 EU/ml.

The enzyme was then further purified by gel permeation chromatography. In this regard, a column containing 850 ml of SEPHACRYL S400 HIGH RESOLUTION (PHARMACIA) was equilibrated with a buffer containing 50 mM KH2PO4/K2HPO4 (pH = 7.0) and then loaded with the remainder of the 6 ml suspension described above, and eluted with the buffer containing 50 mM KH2PO4/K2HPO4 (pH = 7.0), at a flow rate of 1 ml/minute. Respective fractions were then obtained.

The respective fractions containing the highest phenol oxidizing enzyme activities were pooled together, providing a 60 ml suspension containing the purified phenol oxidizing enzyme.

The phenol oxidizing enzyme activity of the suspension was then measured based on the oxidation of ABTS by oxygen. The enzyme activity so measured was 390 EU/ml. *Stachybotrys* chartarum phenol oxidizing enzyme prepared as disclosed above was subjected to SDS polyacrylamide gel electrophoresis and isolated. The isolated fraction was treated with urea and iodoacetamide and digested by the enzyme endoLysC. The fragments resulting from the endoLysC digestion were separated via HPLC (reverse phase monobore C18 column, CH3CN gradient) and collected in a multititer plate. The fractions were analysed by MALDI for mass determination and sequenced via Edman degradation. The following amino acid sequences were determined and are shown in amino terminus to carboxy terminus orientation:

N' DYYFPNYQSARLLXYHDHA C' (SEQ ID NO: 10)

N' RGQVMPYESAGLK C' (SEQ ID NO: 11)

Two degenerated primers were designed based on the peptide sequence. Primer 1 contained the following sequence: TATTACTTTCCNAAYTAYCA (SEQ ID NO: 12) where N represents a mixture of all four nucleotides (A, T, C and G) and Y represents a mixture of T and C only. Primer 2 contained the following sequence: TCGTATGGCATNACCTGNCC (SEQ ID NO: 13).

For isolation of genomic DNA encoding phenol oxidizing enzyme, DNA isolated from *Stachybotrys* chartarum (MUCL # 38898) was used as a template for PCR. The DNA was diluted 100 fold with Tris-EDTA buffer to a final concentration of 88 ng/ul. Ten microliter of diluted DNA was added to the reaction mixture which contained 0.2 mM of each nucleotide (A, G. C and T), 1x reaction buffer, 0.296 microgram of primer 1 and 0.311 microgram of primer 2 in a total of 100 microliter reaction. After heating the mixture at 100 °C for 5 minutes, 2.5 units of Taq DNA polymerase was added to the reaction mix. The PCR reaction was performed at 95 °C for 1 minute, the primers were annealed to the template at 45 °C for 1 minute and extension was done at 68 °C for 1 minute. This cycle was repeated 30 times to achieve a gel-visible PCR fragment. The PCR fragment detected by agarose gel contained a fragment of about 1 kilobase which was then cloned into the plasmid vector pCR-II (Invitrogen). The 1 kb insert was then subjected to nucleic acid sequencing. The sequence data revealed that it was the gene encoding *Stachybotrys* chartarum because the deduced peptide sequence matched the peptide sequences disclosed above sequenced via Edman degradation. The PCR fragments containing the 5' gene and 3' gene were then isolated and sequenced. Figure 1 provides the full length genomic sequence (SEQ ID NO:1) of *Stachybotrys* oxidase including the promoter and terminator sequences. - -

B. Please replace Example III with the following:

- - **Example III**

In this example a shake flask pulp bleaching protocol was used to determine the activity of phenol oxidizing enzymes.

The buffer used is 50 mM Na Acetate, pH 5 or 50mM Tris pH 8.5. Softwood , oxygen delignified pulp with a of kappa 17.3 is used. The enzyme is dosed at 10 ABTS units per g of pulp. The assay can be performed with and without mediators, such as those described infra.

250 ml of pre-warmed buffer is placed in a graduated cylinder. 10 g of wet pulp (at 72% moisture = 2.8 g dry pulp) is placed into a standard kitchen blender with ~120 ml buffer. The pulp is blended on the highest setting for about 30 seconds. The resulting slurry is placed into a large-mouth shake flask (residual pulp is rinsed out of the blender with remaining buffer and spatula) which results in about a 1% consistency in the flask (2.8g/250ml).

The enzyme +/- mediator is added and controls without enzyme are included in the assay. The opening of the flask is covered with 2 thickness cheese cloth and secured with a rubber band. The flasks are placed into a shaker and incubated for 2 hours at ~55°C and 350 rpm.

At the end of the incubation time, 500 mls of 2% NaOH are added directly into the flasks and the shaker temperature is set to 70°C and allowed to incubate for 1.5 hours at 250 rpm. The flask contents are filtered through buchner funnels. The pulp slurries are poured directly into the funnels, without vacuum and are allowed to slowly drip which sets up a filter layer inside the funnel.

Once most of the flask contents are in the funnel, a light vacuum is applied to pull the material into a cake inside the funnel. The filtrate (liquid) is poured back into the original shake flask and swirled to wash residual pulp from the sides. The filtrate is poured back on top of the filter cake. The end result is a fairly clear light golden colored filtrate with most of the pulp caught in the funnel. The filter cake is washed without vacuum, by gently pouring 1 liter of DI water over the filter cake and letting it drip through on its own. A vacuum is applied only at the end to suck the cake dry. The filter cakes are dried in the funnels overnight in a 100°C oven. The dried pulp is manually scraped from the cooled funnels the next day. Microkappa determinations based on the method of the Scandinavian Pulp, Paper and Board Testing committee Scan-c 1:77 (The Scandinavian Pulp, Paper and Board Testing committee Box 5604,S-114, 86 Stockholm, Sweden) are performed to determine % delignification. - -

C. Please replace Example IV with the following:

- - **Example IV**

Example IV describes the Southern hybridization technique used to identify homologous genes from other organisms.

The genomic DNA from several fungal strains including the *Stachybotrys* chartarum, Myrothecium verruvaria, Myrothecium cinctum, Curvalaria *pallescens*, Humicola insolens, Pleurotus eryngii, Pleurotus abalous, Aspergillus niger, Corpinus cineras, Mycellophthora thermophila, Trichoderma reesei, Trametes vesicolor, Chaetomium sp, and Bipolaris *spicifera* was isolated. All fungal species were grown in either CSL medium (described in Dunn-Coleman et al., 1991, Bio/Technology 9:976-981) or MB medium (glucose 40g/l; soytone 10g/l; MB trace elements 1ml/L at pH 5.0) for 2 to 4 days. The mycelia were harvested by filtering through Mirocloth (Calbiochem). The genomic DNA was extracted from cells by repeated phenol/chloroform extraction according to the fungal genomic DNA purification protocol (Hynes MJ, Corrick CM, King JA 1983, Mol Cell Biol 3:1430-1439). Five micrograms genomic DNA were digested with restriction enzyme EcoRI or Hind III overnight at 37 °C and the DNA fragments were separated on 1% agarose gel by electrophoresis in TBE buffer. The DNA fragments were then transferred from agarose gel to the Nitrocellulose membrane in 20XSSC buffer. The probe used for Southern analysis was isolated from plasmids containing either the entire coding region of the *Stachybotrys* phenol oxidizing enzyme (SEQ ID NO:1) or a DNA fragment generated through PCR reaction that covers the internal part of the genes of more than 1 kb in size. The primers used to generate the PCR fragment were Primer 1 containing the following sequence: TATTACTTTCCNAAYTAYCA (SEQ ID NO: 12) where N represents a mixture of all four nucleotides (A, T, C and G) and Y represents a mixture of T and C only and Primer 2 containing the following sequence: TCGTATGGCATNACCTGNCC (SEQ ID NO: 13). Southern hybridizations were performed for 18 to 20 hours at 37 °C in an intermediate stringency hybridization buffer containing 25% formamide, 5x SSPE, 0.5% SDS and 50 ug/ml of sheared Herring DNA. The blots were washed once at 50 °C for 30 minutes in presence of 1 x SSC and 0.1% SDS and washed again at 50 °C for 30 minutes in 0.5x SSC and 0.1% SDS. The Southern blots were exposed to x-ray film for more than 20 hours and up to 3 days. Figures 6, 7, and 8 show that the genomic DNAs of several fungal species contained sequences that were able to hybridize under the conditions

described above to the nucleic acid encoding the *Stachybotrys* phenol oxidizing enzyme shown in SEQ ID NO:1. These fungal species giving the strongest signal (which may indicate a higher identity to the nucleic acid probe than those giving a weaker signal) are Myrothecium verrucaria, Curvalaria *pallescens*, Chaetomium sp, Bipolaris *spicifera*, and Amerosporium *atrum*. Fungal species also hybridizing to nucleic acid encoding the *Stachybotrys* phenol oxidizing enzyme were detected from genomic DNA of Humicola insolens, Pleurotus abalonus, Trichoderma reesei and Mycellophthora thermophila. - -

D. Please replace Example V with the following:

- - **Example V**

Example V describes the cloning of genes encoding fungal enzymes capable of hybridizing to *Stachybotrys* phenol oxidizing enzyme of SEQ ID NO:1.

A. Bipolaris *spicifera*

Based on the DNA and protein sequences comparison of the phenol oxidizing enzyme of SEQ ID NO:1 (from the *Stachybotrys* chartarum) and bilirubin oxidase from the Myrothecium verruvaria (GenBank number 14081), a set of oligonucleotide primers was designed to isolate related sequences from a number of different organisms via hybridization techniques. The following oligonucleotide primers (primer 1: 5' TGGTACCAYGAYCAYGCT 3' (SEQ ID NO: 14) and primer 2: 5' RGACTCGTAKGGCATGAC 3' (SEQ ID NO: 15) (where the Y is an equal mixture of nucleotides T and C, R is an equal mixture of nucleotides A and G and K represents an equal mixture of nucleotides T and G) were used to clone a phenol oxidizing enzyme from Bipolaris *spicifera*. The genomic DNA isolated from Bipolaris *spicifera* was diluted 10 fold with Tris-EDTA buffer to a final concentration of 63 ng/ul. Ten microliters of diluted DNA were added to a reaction mixture which contained 0.2 mM of each nucleotide (A, G. C and T), 1x reaction buffer (10mM Tris, 1.5 mM MgCl2, 50 mM KCl at pH8.3) in a total of 100 microliters reaction in the presence of primers 1 and 2. After heating the mixture at 100 °C for 5 minutes, 2.5 units of Taq DNA polymerase was added to the reaction mix. The PCR reaction was performed at 95 °C for 1 minute, the primer was annealed to the template at 50 °C for 1 minute and extension was done at 72 °C for 1 minute. This cycle was repeated 30 times to achieve a gel-visible PCR

fragment and an extension at 72 °C for 7 minutes was added after 30 cycles. The PCR fragment detected by agarose gel contained a fragment of about 1 kilobase which was then cloned into the plasmid vector pCR-II (Invitrogen). The 1 kb insert was then subjected to nucleic acid sequencing. The 3' end of the gene was isolated by RS-PCR method (Sarkar et al., 1993, PCR Methods and Applications 2:318-322) from the genomic DNA of the Bipolaris *spicifera*. The PCR fragment was cloned into the plasmid vector pCR-II (Invitrogen) and sequenced. The 5' end of the gene was isolated by the same RS-PCR method (Sarkar et al 1993, PCR methods and applications 2:318-322) from the genomic DNA of the Bipolaris *spicifera*. The PCR fragment was also cloned into the plasmid vector pCR-II (Invitrogen) and sequenced. The full length genomic DNA (SEQ ID NO:3) including the regulatory sequence of the promoter and terminator regions is shown in Figure 2 and the amino acid sequence translated from genomic DNA is shown in Figure 3 (SEQ ID NO:4). The sequence data comparison, shown in Figure 4, revealed that it encodes a phenol oxidizing enzyme having about 60.8% identity to the *Stachybotrys* chartarum phenol oxidizing enzyme shown in SEQ ID NO:2 (as determined by MegAlign Program from DNAstar (DNASTAR, Inc. Madison, WI 53715) by Jotun Hein Method (1990, Method in Enzymology, 183: 626-645) with a gap penalty = 11, a gap length penalty = 3 and Pairwise Alignment Parameters Ktuple = 2.

B. Curvularia *pallescens*

Based on the comparison of the nucleic acid and protein sequences of the phenol oxidizing enzyme of SEQ ID NO:1 (obtainable from *Stachybotrys* chartarum) and bilirubin oxidase obtainable from Myrothecium verruvaria (GenBank accession number 14081), a set of oligonucleotide primers was designed to isolate related sequences from a number of different organisms via hybridization techniques. The following oligonucleotide primers (primer 1: 5' TGGTACCAYGAYCAYGCT 3' (SEQ ID NO: 14) and primer 2: 5' TCGTGGATGARRTTGTGRCAR 3' (SEQ ID NO: 16) (where the Y is an equal mixture of nucleotides T and C, R is an equal mixture of nucleotides A and G) were used to clone a phenol oxidizing enzyme from Curvularia *pallescens*. The genomic DNA isolated from Curvularia *pallescens* was diluted with Tris-EDTA buffer to a final concentration of 200 ng/ul. Ten microliters of diluted DNA were added to a reaction mixture which contained 0.2 mM of each nucleotide (A, G. C and T), 1x reaction buffer (10mM Tris, 1.5 mM MgCl2, 50 mM KCl at pH8.3) in a total of 100 microliters reaction in

the presence of primers 1 and 2. After heating the mixture at 100 °C for 5 minutes, 2.5 units of Taq DNA polymerase were added to the reaction mix. The PCR reaction was performed at 95 °C for 1 minute, the primer was annealed to the template at 50 °C for 1 minute and extension was done at 72 °C for 1 minute. This cycle was repeated 30 times and an extension at 72 °C for 7 minutes was added after 30 cycles. The PCR fragment detected by agarose gel contained a fragment of about 900 base pairs. The 900 bp PCR fragment was then subjected to nucleic acid sequencing. The 5' and part of 3'end of the genomic DNA was isolated by inverse PCR method (Triglia T et al, Nucleic Acids Res. 16:8186) from the genomic DNA of Curvularia *pallescens* using two pairs of oligonucleotides based on sequence data from the 900 bp PCR fragment. The full length genomic DNA (SEQ ID NO:6) from the translation start site to the translation stop site is shown in Figure 9 and the putative amino acid sequence translated from genomic DNA is shown in Figure 10 (SEQ ID NO:7). The sequence data comparison, shown in Figure 11, illustrates that the phenol oxidizing enzyme obtainable from Curvularia *pallescens* and having SEQ ID NO:7 has 92.8% identity to the phenol oxidizing enzyme cloned from Bipolaris *spicifera* shown in SEQ ID NO:4 (as determined by MegAlign Program from DNAstar (DNASTAR, Inc. Madison, WI 53715) by Jotun Hein Method (1990, Method in Enzymology, 183: 626-645) with a gap penalty = 11, a gap length penalty = 3 and Pairwise Alignment Parameters Ktuple = 2. SEQ ID NO:7 has 60.8% identity to the *Stachybotrys* oxidase phenol oxidizing enzyme A shown in SEQ ID NO: 2.

C. Amerosporium *atrum*

Based on the DNA and protein sequences comparison of the phenol oxidizing enzyme of SEQ ID NO:1 (from the *Stachybotrys* chartarum) and bilirubin oxidase from the Myrothecium verruvaria (GenBank number 14081), a set of oligonucleotide primers was designed to isolate related sequences from a number of different organisms via hybridization techniques. The following oligonucleotide primers (primer 1: 5' TGGTACCAYGAYCAYGCT 3' (SEQ ID NO: 14) and primer 2: 5' CXAGACRACRTCYTTRAGACC 3' (SEQ ID NO: 17) (where the Y is an equal mixture of nucleotides T and C, R is an equal mixture of nucleotides A and G and X is an equal mixture of nucleotides G, A, T and C) were used to clone a phenol oxidizing enzyme from Amerosporium *atrum* . A reaction mixture which contained 0.2 mM of each nucleotide (A, G. C and T), 1x reaction buffer (10mM Tris, 1.5 mM MgCl2, 50 mM KCl at

pH8.3), 1ul of 50 pmol/ul primers 1 and 2 in a total of 50 microliters reaction were added to a hot start tube (Molecular Bio-Products). The mixture was heated to 95 °C for 90 seconds , and the tubes were cooled on ice for 5 minutes. The genomic DNA isolated from Amerosporium *atrum* was diluted 10 fold with Tris-EDTA buffer to a final concentration of 41 ng/ul. About 1 ul of the diluted DNA was added to the hot start tube with 1x reaction buffer (10mM Tris, 1.5 mM MgCl2, 50 mM KCl at pH8.3), 2.5 units of Taq DNA polymerase in a total volume to 50 microliters. The reaction mixture was heated to 95 °C for 5 minutes. The PCR reaction was performed at 95 °C for 1 minute, the primer was annealed to the template at 51°C for 1 minute and extension was done at 72 °C for 1 minute. This cycle was repeated 29 times to achieve a gel-visible PCR fragment and an extension at 72 °C for 7 minutes was added after 29 cycles. The PCR fragment detected by agarose gel contained a fragment of about 1 kilobase. The 1 kb insert was then subjected to nucleic acid sequencing. The genomic sequence for the Amerosporium *atrum* is shown in Figure 13. An amino acid alignment of the amino acid obtainable from Amerosporium *atrum* and SEQ ID NO: 2 is shown in Figure 14. - -

In The Claims:

Please replace the following amended claims with the clean copy of said claims as presented below. A marked-up version of the amended claims is attached hereto as Appendix II. Additionally, a copy of the status of the claims is attached as Appendix III.

1.(Once amended) An isolated oxidoreductase enzyme encoded by a nucleic acid capable of hybridizing to the nucleic acid having the sequence as shown in SEQ ID NO:1 or a fragment thereof, under conditions of high stringency which includes hybridization at about 37°C in buffer including 50% formamide and washing at about 65°C, said isolated oxidoreductase having at least 80% sequence identity to the amino acid sequence of SEQ ID NO: 2, wherein said isolated oxidoreductase enzyme is obtainable from a source other than *Stachybotrys chartarum* .

2.(Once amended) The oxidoreductase enzyme of Claim 1 having at least 85% identity to the oxidoreductase enzyme having the amino acid sequence as disclosed in SEQ ID NO:2.

3.(Once amended) The oxidoreductase enzyme of Claim 1, wherein said enzyme is obtained from a bacteria, yeast or non-*Stachybotrys* fungus.

4.(Once amended) The oxidoreductase enzyme of Claim 3 wherein said fungus is selected from the group consisting of Myrothecium species, Curvularia species, Chaetomium species, Bipolaris species, Humicola species, Pleurotus species, Trichoderma species, Mycellophthora species and Amerosporium species.

5.(Once amended) The oxidoreductase enzyme of Claim 4 wherein the fungus are selected from the group consisting of Myrothecium verrucaria, Curvalaria *pallescens*, Chaetomium sp, Bipolaris *spicifera*, Humicola insolens, Pleurotus abalonus, Trichoderma reesei, Mycellophthora thermophila and Amerosporium *atrum*.

6.(Once amended) The oxidoreductase enzyme of Claim 4 wherein said fungus is a Bipolaris species, a Curvularia species or a Amerosporium species.

7. (Once amended) The oxidoreductase enzyme of Claim 6 wherein said fungus is Bipolaris *spicifera*, Curvularia *pallescens* or Amerosporium *atrum*.

8.(Once Amended) An isolated oxidoreductase enzyme comprising an amino acid sequence having at least 95% identity to the amino acid sequence disclosed in SEQ ID NO: 4, SEQ ID NO: 7 or SEQ ID NO: 9.

9.(Once amended) An isolated polynucleotide encoding the amino acid comprising the sequence as shown in SEQ ID NO:7 or SEQ ID NO:9.

11.(Once amended) The isolated polynucleotide of Claim 9 comprising the nucleic acid sequence as disclosed in SEQ ID NO:6 or SEQ ID NO:8.

12.(Once amended) An isolated polynucleotide encoding an oxidoreductase enzyme that hybridizes to the sequence shown in SEQ ID NO: 3, SEQ ID NO:6 or SEQ ID NO:8 or a fragment thereof, under conditions of intermediate stringency which includes hybridization at about 37°C in buffer including 25% formamide and washing at about 50°C.

16.(Once amended) The host cell of Claim 15 wherein said filamentous fungus is selected form the group consisting of Aspergillus species, Trichoderma species and Mucor species.

18.(Once amended) The host cell of Claim 17 wherein said yeast is selected from the group consisting of Saccharomyces, Pichia, Schizosaccharomyces, Hansenula, Kluyveromyces, and Yarrowia species.

20.(Once amended) The host cell of Claim 19 wherein said bacterium is selected from the group consisting of Bacillus and Escherichia species.

21.(Once amended) A method for producing an oxidoreductase enzyme in a host cell comprising the steps of:

a) obtaining a host cell comprising a polynucleotide that hybridizes to the nucleic acid shown in SEQ ID NO:1, or a fragment thereof, under conditions of intermediate stringency which includes hybridization at about 37° C in buffer including 25% formamide and washing at about 50°C; and

b) growing said host cell under conditions suitable for the production of said oxidoreductase enzyme.

23.(Once amended) The method of Claim 22 wherein the fungus is selected from the group consisting of Myrothecium verrucaria, Curvalaria *pallescens*, Chaetomium sp, Bipolaris *spicifera*, Humicola insolens, Pleurotus abalonus, Trichoderma reesei, Mycellophthora thermophila or Amerosporium *atrum*.

24.(Once amended) The method of Claim 21 wherein the oxidoreductase sequence comprises the amino acid sequence as disclosed in SEQ ID NO:7 or SEQ ID NO:9.

25.(Once amended) The method of Claim 21 wherein said polynucleotide comprises the sequence as shown in SEQ ID NO: 6 or SEQ ID NO: 8.

26.(Once amended) The method of Claim 21 wherein said host cell includes filamentous fungus, yeast or bacteria.

27.(Once amended) The method of Claim 26 wherein said yeast is selected from the group consisting of Saccharomyces, Pichia, Schizosaccharomyces, Hansenula, Kluyveromyces, and Yarrowia species.

28. (Once amended) The method of Claim 26 wherein said filamentous fungus is selected from the group consisting of Aspergillus species, Trichoderma species and Mucor species.

29.(Once amended) A method for producing a host cell comprising an oxidoreductase enzyme comprising the steps of:

a) obtaining a polynucleotide that hybridizes to the nucleic acid having the sequence as shown in SEQ ID NO:1, or a fragment thereof, under conditions of intermediate stringency which includes hybridization at about 37°C in buffer including 25% formamide and washing at about 50°C;

b) introducing said polynucleotide into said host cell; and

c) growing said host cell under conditions suitable for the production of said oxidoreductase enzyme.

30.(Once amended) The method of Claim 29 wherein said host cell includes filamentous fungus, yeast or bacteria.

31.(Once amended) The method of Claim 30 wherein said filamentous fungus is selected from the group consisting of Aspergillus species, Trichoderma species and Mucor species.

34.(Once amended) The method of Claim 33 wherein said polynucleotide comprises the nucleic acid sequence as shown in SEQ ID NO:6 or SEQ ID NO:8.

Please add the following new claims

35. The oxidoreductase enzyme of Claim 2, wherein said enzyme has at least 90% identity to the amino acid sequence of SEQ ID NO: 2.

36. The isolated oxidoreductase of Claim 8, wherein said oxidoreductase enzyme has an amino acid sequence having at least 95% identity to the sequence disclosed in SEQ ID NO: 4

37. The isolated oxidoreductase enzyme of Claim 8, wherein said oxidoreductase enzyme has an amino acid sequence having at least 95% identity to the sequence disclosed in SEQ ID NO: 7

38. The isolated oxidoreductase enzyme of Claim 8, wherein said oxidoreductase enzyme has an amino acid sequence having at least 95% identity to the sequence disclosed in SEQ ID NO: 9.

39. An isolated polynucleotide encoding the oxidoreductase enzyme of Claim 8.

40. An expression vector comprising the polynucleotide of Claim 9.

41. A host cell comprising the expression vector of Claim 40.

42. The host cell of Claim 41 that is a filamentous fungus.

43. The host cell of Claim 42 wherein said filamentous fungus is selected form the group consisting of Aspergillus species, Trichoderma species and Mucor species.

44. The host cell of Claim 41 that is a yeast.

45. The host cell of Claim 44 wherein said yeast is selected from the group consisting of Saccharomyces, Pichia, Schizosaccharomyces, Hansenula, Kluyveromyces, and Yarrowia species.

46. The host cell of Claim 41 wherein said host is a bacterium.

47. The host cell of Claim 46 wherein said bacterium is a *Bacillus* species.

48. The method for producing an oxidoreductase enzyme according to Claim 21 further comprising recovering said oxidoreductase from step b).

49. A method for producing an oxidoreductase enzyme in a host cell comprising the steps of:

a) obtaining a host cell comprising a polynucleotide that hybridizes to the nucleic acid shown in SEQ ID NO:1, or a fragment thereof, under conditions of high stringency which includes hybridization at about 37°C in buffer including 50% formamide and washing at about 65°C, and

b) growing said host cell under conditions suitable for the production of said oxidoreductase enzyme.

50. The method of Claim 49 further comprising recovering said oxidoreductase from step b).

REMARKS

This application is being filed as a continuation application of parent application serial number 09/ 468,578 filed December 21, 1999, which is a continuation in part application of application serial number 09/338, 723 filed June 23, 1999, which is a continuation application of application serial number 09/220,871, filed December 23, 1998, now abandoned.

Applicants have amended the specification to include sequence identifiers and correct minor grammatical and spelling mistakes. A marked-up version of the amended sections is appended hereto. Additionally, the original abstract has been replaced with an abstract that is one paragraph.

With entry of the instant amendment claims 1 - 50 are pending. Claims 1 - 9, 11, 12, 16, 18, 20, 21, 23 - 31 and 34 have been amended, and claims 35 - 50 are new. New matter has not been added by the instant amendment.

In general, Applicants have used the term "oxidoreductase" enzyme instead of the "phenol oxidizing" enzyme in the claims, and reference is made to claims 1 - 8, 12, 21, 24, 29 and new claims 35 - 39, 48 and 49. It is a basic understanding of one of ordinary skill in the art that the phenol oxidizing enzymes as defined by Applicants are oxidoreductase enzymes. Therefore, it is without prejudice that Applicants have used this term to define the enzymes of the invention. (Reference is made to Enzyme Nomenclature, 1992, Academic Press, International Union of Biochemistry and Molecular Biology). Each of the classes of enzymes as cited on page 9 of the specification (EC1.10.3.2; EC1.3.3.4: EC1.14.18.1 and EC1.10.3.1) are oxidoreductase enzymes.

Claim 1 has been amended to recite high stringency including certain conditions and wherein the oxidoreductase enzyme has at least 80% identity to SEQ ID NO: 2 and wherein SEQ ID NO: 2 is obtained from a source other than *Stachybotrys chartarum*. Support is found at page 13, lines 2 - 6, page 7, lines 17 - 20 and page 2 lines 14 - 20 of the specification. Amended dependent claim 2 recites 85% sequence identity, and support is found at page 2, lines 14 - 20. Claims 3 - 7, 9, 11, 16, 18, 20, 23 - 28, 30, 31 and 34 have been amended to correct spelling, grammar and claim format. Specifically Markush language has been incorporated into some of the claims, and recitation of SEQ ID NO: 3 or SEQ ID NO: 4 has been omitted in some of the claims. Claim 8 has been amended to include an enzyme having 95% identity to SEQ ID NO: 4, SEQ ID NO: 7 or SEQ ID NO: 9. Support is found at page 10, lines 12 - 29 of the specification. Claims

12, 21 and 29 now include intermediate hybridization conditions. Support for intermediate hybridization conditions is found at page 13, lines 7 - 10. Optionally step c) has been omitted from claim 21 and incorporated into new dependent claim 48.

New claim 35 depends from claim 2 and further defines the % identity as 90%. Claims 36 - 38 depend from claim 8 and recite each embodiment of claim 8. Dependent claims 39 - 47 are directed to a) polynucleotides, encoding the enzyme as claimed in claim 8, b) expression vectors including the polynucleotides and c) host cells. Support is found in the original claims. Claim 48 recites the additional step of recovering the oxidoreductase of claim 21. New independent claim 49 is directed to a method of producing an oxidoreductase enzyme under high stringency conditions, and support is found in original claim 21. Claim 50 depends from claim 49 and defines a further step of recovering the oxidoreductase enzyme.

It is respectfully asserted that pending claims 1 - 50 are patentable. Allowance of said claims is kindly solicited.

Respectfully submitted,

Date: February 19, 2002

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Enclosures: Marked-up version of the specification
Marked-up version of claims
Status of claims

MARKED-UP VERSION OF THE SPECIFICATION

B. Starting at page 21 - page 22 of Example I please amend the specification as follows:

- - The phenol oxidizing enzyme activity of the suspension was then measured using the standard assay procedure, based on the oxidation of ABTS by oxygen, as was described above (but with the exception that the preparation being assayed is the resuspended concentration and not the supernatant dilutions). The phenol oxidizing enzyme activity so measured was 5200 EU/ml.

The enzyme was then further purified by gel permeation chromatography. In this regard, a column containing 850 ml of SEPHACRYL S400 HIGH RESOLUTION (PHARMACIA) was equilibrated with a buffer containing 50 mM KH2PO4/K2HPO4 (pH = 7.0) and then loaded with the remainder of the 6 ml suspension described above, and eluted with the buffer containing 50 mM KH2PO4/K2HPO4 (pH = 7.0), at a flow rate of 1 ml/minute. Respective fractions were then obtained.

The respective fractions containing the highest phenol oxidizing enzyme activities were pooled together, providing a 60 ml suspension containing the purified phenol oxidizing enzyme.

The phenol oxidizing enzyme activity of the suspension was then measured based on the oxidation of ABTS by oxygen. The enzyme activity so measured was 390 EU/ml. *Stachybotrys* chartarum phenol oxidizing enzyme prepared as disclosed above was subjected to SDS polyacrylamide gel electrophoresis and isolated. The isolated fraction was treated with urea and iodoacetamide and digested by the enzyme endoLysC. The fragments resulting from the endoLysC digestion were separated via HPLC (reverse phase monobore C18 column, CH3CN gradient) and collected in a multititer plate. The fractions were analysed by MALDI for mass determination and sequenced via Edman degradation. The following amino acid sequences were determined and are shown in amino terminus to carboxy terminus orientation:

N' DYYFPNYQSARLLXYHDHA C' **(SEQ ID NO: 10)**

N' RGQVMPYESAGLK C' **(SEQ ID NO: 11)**

Two degenerated primers were designed based on the peptide sequence. Primer 1 contain**[s]ed** the following sequence: TATTACTTTCCNAAYTAYCA **(SEQ ID NO: 12)** where N represents a mixture of all four nucleotides (A, T, C and G) and Y represents a mixture of T and C only. Primer 2 contain**[s]ed** the following sequence: TCGTATGGCATNACCTGNCC **(SEQ ID NO: 13)**.

For isolation of genomic DNA encoding phenol oxidizing enzyme, DNA isolated from *Stachybotrys* chartarum (MUCL # 38898) was used as a template for PCR. The DNA was diluted 100 fold with Tris-EDTA buffer to a final concentration of 88 ng/ul. Ten microliter of diluted DNA was added to the reaction mixture which contained 0.2 mM of each nucleotide (A, G. C and T), 1x reaction buffer, 0.296 microgram of primer 1 and 0.311 microgram of primer 2 in a total of 100 microliter reaction. After heating the mixture at **[100oC] 100 °C** for 5 minutes, 2.5 units of Taq DNA polymerase was added to the reaction mix. The PCR reaction was performed at **[95oC] 95 °C** for 1 minute, the primers were annealed to the template at **[45oC] 45 °C** for 1 minute and extension was done at **[68oC] 68 °C** for 1 minute. This cycle was repeated 30 times to achieve a gel-visible PCR fragment. The PCR fragment detected by agarose gel contained a fragment of about 1 kilobase which was then cloned into the plasmid vector pCR-II (Invitrogen). The 1 kb insert was then subjected to nucleic acid sequencing. The sequence data revealed that it was the gene encoding *Stachybotrys* chartarum because the deduced peptide sequence matched the peptide sequences disclosed above sequenced via Edman degradation. The PCR fragments containing the 5' gene and 3' gene were then isolated and sequenced. Figure 1 provides the full length genomic sequence (SEQ ID NO:1) of *Stachybotrys* oxidase including the promoter and terminator sequences. - -

C. Please replace Example III with the following:

- - **Example III**

[This] **In this** example a shake flask pulp bleaching protocol **was** used to determine the activity of phenol oxidizing enzymes.

The buffer used is 50 mM Na Acetate, pH 5 or 50mM Tris pH 8.5. Softwood , oxygen delignified pulp with a of kappa 17.3 is used. The enzyme is dosed at 10 ABTS units per g of pulp. The assay can be performed with and without mediators, such as those described infra.

250 ml of pre-warmed buffer is placed in a graduated cylinder. 10 g of wet pulp (at 72% moisture = 2.8 g dry pulp) is placed into a standard kitchen blender with ~120 ml buffer. The pulp is blended on the highest setting for about 30 seconds. The resulting slurry is placed into a large-mouth shake flask (residual pulp is rinsed out of the blender with remaining buffer and spatula) which results in about a 1% consistency in the flask (2.8g/250ml).

The enzyme +/- mediator is added and controls without enzyme are included in the assay. The opening of the flask is covered with 2 thickness cheese cloth and secured with a rubber band. The flasks are placed into a shaker and incubated for 2 hours at ~55°C and 350 rpm.

At the end of the incubation time, 500 mls of 2% NaOH are added directly into the flasks and the shaker temperature is set to 70°C and allowed to incubate for 1.5 hours at 250 rpm. The flask contents are filtered through buchner funnels. The pulp slurries are poured directly into the funnels, without vacuum and are allowed to slowly drip which sets up a filter layer inside the funnel.

Once most of the flask contents are in the funnel, a light vacuum is applied to pull the material into a cake inside the funnel. The filtrate (liquid) is poured back into the original shake flask and swirled to wash residual pulp from the sides. The filtrate is poured back on top of the filter cake. The end result is a fairly clear light golden colored filtrate with most of the pulp caught in the funnel. The filter cake is washed without vacuum, by gently pouring 1 liter of DI water over the filter cake and letting it drip through on its own. A vacuum is applied only at the end to suck the cake dry. The filter cakes are dried in the funnels overnight in a 100°C oven. The dried pulp is manually scraped from the cooled funnels the next day. Microkappa determinations based on the method of the Scandinavian **[Pulp ,Paper]** **Pulp, Paper** and Board Testing committee Scan-c 1:77 (The Scandinavian **[Pulp ,Paper]** **Pulp, Paper** and Board Testing committee Box 5604,S-114, 86 Stockholm, Sweden) are performed to determine % delignification. - -

D. Please replace Example IV with the following:

- - **Example IV**

Example IV describes the Southern hybridization technique used to identify homologous genes from other organisms.

The genomic DNA from several fungal strains including the *Stachybotrys* chartarum, Myrothecium verruvaria, Myrothecium cinctum, Curvalaria *pallescens*, Humicola **[insulas]** **insolens**, Pleurotus eryngii, Pleurotus abalous, Aspergillus niger, Corpinus cineras, Mycellophthora thermophila, Trichoderma reesei, Trametes vesicolor, Chaetomium sp, and Bipolaris *spicifera* was isolated. All fungal species were grown in either CSL medium (described in Dunn-Coleman et al., 1991, Bio/Technology 9:976-981) or MB medium (glucose 40g/l; soytone 10g/l; MB trace elements 1ml/L at pH 5.0) for 2 to 4 days. The mycelia were harvested by filtering through Mirocloth (Calbiochem). The genomic DNA was extracted from cells by repeated phenol/chloroform extraction according to the fungal genomic DNA purification protocol (Hynes MJ, Corrick CM, King JA 1983, Mol Cell Biol 3:1430-1439). Five micrograms genomic DNA were digested with restriction enzyme EcoRI or Hind III overnight at **[37oC]** **37 °C** and the DNA fragments were separated on 1% agarose gel by electrophoresis in TBE buffer. The DNA fragments were then transferred from agarose gel to the Nitrocellulose membrane in 20XSSC buffer. The probe used for Southern analysis was isolated from plasmids containing either the entire coding region of the *Stachybotrys* phenol oxidizing enzyme (SEQ ID NO:1) or a DNA fragment generated through PCR reaction that covers the internal part of the genes of more than 1 kb in size. The primers used to generate the PCR fragment were Primer 1 containing the following sequence: TATTACTTTCCNAAYTAYCA **(SEQ ID NO: 12)** where N represents a mixture of all four nucleotides (A, T, C and G) and Y represents a mixture of T and C only and Primer 2 containing the following sequence: TCGTATGGCATNACCTGNCC **(SEQ ID NO: 13)**. Southern hybridizations were performed for 18 to 20 hours at **[37oC]** **37 °C** in an intermediate stringency hybridization buffer containing 25% formamide, 5x SSPE, 0.5% SDS and 50 ug/ml of sheared Herring DNA. The blots were washed once at **[50oC]** **50 °C** for 30 minutes in presence of 1 x SSC and 0.1% SDS and washed again at **[50oC]** **50 °C** for 30 minutes in 0.5x SSC and 0.1% SDS. The Southern blots were exposed to x-ray film for more than 20 hours and up to 3 days. Figures 6, 7, and 8 show**[ed]** that the genomic DNAs of several fungal species contained sequences that were able to hybridize under the conditions described above to the nucleic acid encoding the *Stachybotrys* phenol oxidizing enzyme shown in SEQ ID NO:1. These fungal species giving the strongest signal (which may indicate a higher identity to the nucleic acid probe than those giving a weaker signal) are Myrothecium verrucaria, Curvalaria *pallescens*,

Chaetomium sp, Bipolaris *spicifera*, and Amerosporium *atrum*. Fungal species also hybridizing to nucleic acid encoding the *Stachybotrys* phenol oxidizing enzyme were detected from genomic DNA of Humicola insolens, Pleurotus abalonus, Trichoderma reesei and Mycellophthora thermophila. - -

E. Please replace Example V with the following.

- - **Example V**

Example V describes the cloning of genes encoding fungal enzymes capable of hybridizing to *Stachybotrys* phenol oxidizing enzyme of SEQ ID NO:1.

A. Bipolaris *spicifera*

Based on the DNA and protein sequences comparison of the phenol oxidizing enzyme of SEQ ID NO:1 (from the *Stachybotrys* chartarum) and bilirubin oxidase from the Myrothecium verruvaria (GenBank number 14081), a set of oligonucleotide primers was designed to isolate related sequences from a number of different organisms via hybridization techniques. The following oligonucleotide primers (primer 1: 5' TGGTACCAYGAYCAYGCT 3' **(SEQ ID NO: 14)** and primer 2: 5' RGACTCGTAKGGCATGAC 3' **(SEQ ID NO: 15)** (where the Y is an equal mixture of nucleotides T and C, R is an equal mixture of nucleotides A and G and K represents an equal mixture of nucleotides T and G) were used to clone a phenol oxidizing enzyme from Bipolaris *spicifera*. The genomic DNA isolated from Bipolaris *spicifera* was diluted 10 fold with Tris-EDTA buffer to a final concentration of 63 ng/ul. Ten microliters of diluted DNA were added to a reaction mixture which contained 0.2 mM of each nucleotide (A, G. C and T), 1x reaction buffer (10mM Tris, 1.5 mM MgCl2, 50 mM KCl at pH8.3) in a total of 100 microliters reaction in the presence of primers 1 and 2. After heating the mixture at **[100oC] 100 °C** for 5 minutes, 2.5 units of Taq DNA polymerase was added to the reaction mix. The PCR reaction was performed at **[95oC] 95 °C** for 1 minute, the primer was annealed to the template at **[50oC] 50 °C** for 1 minute and extension was done at **[72oC] 72 °C** for 1 minute. This cycle was repeated 30 times to achieve a gel-visible PCR fragment and an extension at **[72oC] 72 °C** for 7 minutes was added after 30 cycles. The PCR fragment detected by agarose gel contained a

fragment of about 1 kilobase which was then cloned into the plasmid vector pCR-II (Invitrogen). The 1 kb insert was then subjected to nucleic acid sequencing. The 3' end of the gene was isolated by RS-PCR method (Sarkar et al., 1993, PCR Methods and Applications 2:318-322) from the genomic DNA of the Bipolaris *spicifera*. The PCR fragment was cloned into the plasmid vector pCR-II (Invitrogen) and sequenced. The 5' end of the gene was isolated by the same RS-PCR method (Sarkar et al 1993, PCR methods and applications 2:318-322) from the genomic DNA of the Bipolaris *spicifera*. The PCR fragment was also cloned into the plasmid vector pCR-II (Invitrogen) and sequenced. The full length genomic DNA (SEQ ID NO:3) including the regulatory sequence of the promoter and terminator regions is shown in Figure 2 and the amino acid sequence translated from genomic DNA is shown in Figure 3 (SEQ ID NO:4). The sequence data comparison, shown in Figure 4, revealed that it encodes a phenol oxidizing enzyme having about 60.8% identity to the *Stachybotrys* chartarum phenol oxidizing enzyme shown in **[SEQ ID NO:1]** **SEQ ID NO: 2** (as determined by MegAlign Program from DNAstar (DNASTAR, Inc. **[Maidson]** **Madison**, WI 53715) by Jotun Hein Method (1990, Method in Enzymology, 183: 626-645) with a gap penalty = 11, a gap length penalty = 3 and Pairwise Alignment Parameters Ktuple = 2.

B. Curvularia *pallescens*

Based on the comparison of the nucleic acid and protein sequences of the phenol oxidizing enzyme of SEQ ID NO: 1 (obtainable from *Stachybotrys* chartarum) and bilirubin oxidase obtainable from Myrothecium verruvaria (GenBank accession number 14081), a set of oligonucleotide primers was designed to isolate related sequences from a number of different organisms via hybridization techniques. The following oligonucleotide primers (primer 1: 5' TGGTACCAYGAYCAYGCT 3' **(SEQ ID NO: 14)** and primer 2: 5' TCGTGGATGARRTTGTGRCAR 3' **(SEQ ID NO: 16)** (where the Y is an equal mixture of nucleotides T and C, R is an equal mixture of nucleotides A and G) were used to clone a phenol oxidizing enzyme from Curvularia *pallescens*. The genomic DNA isolated from Curvularia *pallescens* was diluted with Tris-EDTA buffer to a final concentration of 200 ng/ul. Ten microliters of diluted DNA were added to a reaction mixture which contained 0.2 mM of each nucleotide (A, G. C and T), 1x reaction buffer (10mM Tris, 1.5 mM MgCl2, 50 mM KCl at pH8.3) in a total of 100 microliters reaction in the presence of primers 1 and 2. After heating the mixture at **[100oC]** **100 °C** for 5

minutes, 2.5 units of Taq DNA polymerase were added to the reaction mix. The PCR reaction was performed at **[95oC]** **95 °C** for 1 minute, the primer was annealed to the template at **[50oC]** **50 °C** for 1 minute and extension was done at **[72oC]** **72 °C** for 1 minute. This cycle was repeated 30 times and an extension at **[72oC]** **72 °C** for 7 minutes was added after 30 cycles. The PCR fragment detected by agarose gel contained a fragment of about 900 base pairs. The 900 bp PCR fragment was then subjected to nucleic acid sequencing. The 5' and part of 3'end of the genomic DNA was isolated by inverse PCR method (Triglia T et al, Nucleic Acids Res. 16:8186) from the genomic DNA of Curvularia *pallescens* using two pairs of oligonucleotides based on sequence data from the 900 bp PCR fragment. The full length genomic DNA (SEQ ID NO:6) from the translation start site to the translation stop site is shown in Figure 9 and the putative amino acid sequence translated from genomic DNA is shown in Figure 10 (SEQ ID NO:7). The sequence data comparison, shown in Figure 11, illustrates that the phenol oxidizing enzyme obtainable from Curvularia *pallescens* and having SEQ ID NO:7 has 92.8% identity to the phenol oxidizing enzyme cloned from Bipolaris *spicifera* shown in SEQ ID NO:4 (as determined by MegAlign Program from DNAstar (DNASTAR, Inc. **[Maidson]** **Madison**, WI 53715) by Jotun Hein Method (1990, Method in Enzymology, 183: 626-645) with a gap penalty = 11, a gap length penalty = 3 and Pairwise Alignment Parameters Ktuple = 2. SEQ ID NO:7 has 60.8% identity to the *Stachybotrys* oxidase phenol oxidizing enzyme A shown in **[SEQ ID NO:1]** **SEQ ID NO: 2**.

C. Amerosporium *atrum*

Based on the DNA and protein sequences comparison of the phenol oxidizing enzyme of SEQ ID NO:1(from the *Stachybotrys* chartarum) and bilirubin oxidase from the Myrothecium verruvaria (GenBank number 14081), a set of oligonucleotide primers was designed to isolate related sequences from a number of different organisms via hybridization techniques. The following oligonucleotide primers (primer 1: 5' TGGTACCAYGAYCAYGCT 3' **(SEQ ID NO: 14)** and primer 2: 5' CXAGACRACRTCYTTRAGACC 3' **(SEQ ID NO: 17)** (where the Y is an equal mixture of nucleotides T and C, R is an equal mixture of nucleotides A and G and X is an equal mixture of nucleotides G, A, T and C) were used to clone a phenol oxidizing enzyme from Amerosporium *atrum* . A reaction mixture which contained 0.2 mM of each

nucleotide (A, G. C and T), 1x reaction buffer (10mM Tris, 1.5 mM MgCl2, 50 mM KCl at pH8.3), 1ul of 50 pmol/ul primers 1 and 2 in a total of 50 microliters reaction were added to a hot start tube (Molecular Bio-Products). The mixture was heated to **[95 C]** **<u>95 °C</u>** for 90 seconds , and the tubes were cooled on ice for 5 minutes. The genomic DNA isolated from Amerosporium *atrum* was diluted 10 fold with Tris-EDTA buffer to a final concentration of 41 ng/ul. About 1 ul of the diluted DNA was added to the hot start tube with 1x reaction buffer (10mM Tris, 1.5 mM MgCl2, 50 mM KCl at pH8.3), 2.5 units of Taq DNA polymerase in a total volume to 50 microliters. The reaction mixture was heated to **[95 C]** **<u>95 °C</u>** for 5 minutes. The PCR reaction was performed at **[95oC]** **<u>95 °C</u>** for 1 minute, the primer was annealed to the template at **[51oC]** **<u>51 °C</u>** for 1 minute and extension was done at **[72oC]** **<u>72 °C</u>** for 1 minute. This cycle was repeated 29 times to achieve a gel-visible PCR fragment and an extension at **[72oC]** **<u>72 °C</u>** for 7 minutes was added after 29 cycles. The PCR fragment detected by agarose gel contained a fragment of about 1 kilobase. The 1 kb insert was then subjected to nucleic acid sequencing. The genomic sequence for the Amerosporium *atrum* is shown in Figure 13. An amino acid alignment of the amino acid obtainable from Amerosporium *atrum* and SEQ ID NO:2 is shown in Figure 14.

Marked-up Version of Amended Claims

1.(Once amended) **[A phenol oxidizing]** **An isolated oxidoreductase** enzyme encoded by a nucleic acid capable of hybridizing to the nucleic acid having the sequence as shown in SEQ ID NO:1 or a fragment thereof, under conditions of high **[to intermediate]** stringency **which includes hybridization at about 37°C in buffer including 50% formamide and washing at about 65°C, said isolated oxidoreductase having at least 80% sequence identity to the amino acid sequence disclosed in SEQ ID NO: 2, wherein said isolated oxidoreductase enzyme is obtainable from a source other than *Stachybotrys chartarum*** .

2.(Once amended) The **[phenol oxidizing]** **oxidoreductase** enzyme of Claim 1 having at least 60% identity to the phenol oxidizing enzyme having the amino acid sequence as disclosed in SEQ ID NO:2.

3.(Once amended) The **[phenol oxidizing]** **oxidoreductase** enzyme of Claim 1, **wherein said enzyme is obtained** **[obtainable]** from a bacteria, yeast or non-*Stachybotrys* fungus.

4.(Once amended) The **[phenol oxidizing]** **oxidoreductase** enzyme of Claim 3 wherein said fungus **[includes]** **is selected from the group consisting of** Myrothecium species, Curvularia species, Chaetomium species, Bipolaris species, Humicola species, Pleurotus species, Trichoderma species, Mycellophthora species and Amerosporium species.

5.(Once amended) The **[phenol oxidizing]** **oxidoreductase** enzyme of Claim 4 wherein the fungus **[include]** **are selected from the group consisting of** Myrothecium verrucaria, Curvalaria *pallescens*, Chaetomium sp, Bipolaris *spicifera*, Humicola insolens, Pleurotus abalonus, Trichoderma reesei, Mycellophthora thermophila and Amerosporium *atrum.*

6.(Once amended) The **[phenol oxidizing]** **oxidoreductase** enzyme of Claim 4 wherein said fungus is a **[Biopolarius]** **Bipolaris** species, a Curvularia species or a Amerosporium species.

7. (Once amended) The **[phenol oxidizing]** **oxidoreductase** enzyme of Claim 6 wherein said fungus is **[Biopolarius]** **Bipolaris** *spicifera*, Curvularia *pallescens* or Amerosporium *atrum*.

8.(Once Amended) **[The phenol oxidizing enzyme of Claim 1]** An isolated oxidoreductase enzyme comprising **[the]** **an** amino acid sequence **having at least 95% identity to the amino acid sequence** **[as]** disclosed in SEQ ID NO:4, SEQ ID NO:7 or SEQ ID NO:9.

9.(Once amended) An isolated polynucleotide encoding the amino acid comprising the sequence as shown in **[SEQ ID NO:4,]** SEQ ID NO:7 or SEQ ID NO:9.

11.(Once amended) The isolated polynucleotide of **[Claim 10]** **claim 9** comprising the nucleic acid sequence as disclosed in **[SEQ ID NO:3,]** SEQ ID NO:6 or SEQ ID NO:8.

12.(Once amended) An isolated polynucleotide encoding **[capable of hybridizing to the polynucleotide comprising]** **an oxidoreductase enzyme that hybridizes to** the sequence **[as]** shown in SEQ ID NO: 3, SEQ ID NO:6 or SEQ ID NO:8 or a fragment thereof, under conditions of intermediate stringency **which includes hybridization at about 37°C in buffer including 25% formamide and washing at about 50°C**.

16.(Once amended) The host cell of Claim 15 wherein said filamentous fungus **[includes]** **is selected form the group consisting of** Aspergillus species, Trichoderma species and Mucor species.

18.(Once amended) The host cell of Claim 17 wherein said yeast **[includes]** **is selected from the group consisting of** Saccharomyces, Pichia, Schizosaccharomyces, Hansenula, Kluyveromyces, and Yarrowia species.

20.(Once amended) The host cell of Claim 19 wherein said bacterium **[includes]** **is selected from the group consisting of** Bacillus and Escherichia species.

21.(Once amended) A method for producing **[a phenol oxidizing]** **an oxidoreductase** enzyme in a host cell comprising the steps of:

a) obtaining a host cell comprising a polynucleotide **[capable of hybridizing]** **that hybridizes** to the nucleic acid **[having the sequence as]** shown in SEQ ID NO:1, or a fragment thereof, under conditions of **[high to]** intermediate stringency **which includes hybridization at about 37 °C in buffer including 25% formamide and washing at about 50°C** ; **and**

b) growing said host cell under conditions suitable for the production of said **[phenol oxidizing]** **oxidoreductase** enzyme**[; and**

c) optionally recovering said phenol oxidizing enzyme produced].

23.(Once amended) The method of Claim 22 wherein the fungus **[includes]** **is selected from the group consisting of** Myrothecium verrucaria, Curvalaria *pallescens*, Chaetomium sp, Bipolaris *spicifera*, Humicola insolens, Pleurotus abalonus, Trichoderma reesei, Mycellophthora thermophila or Amerosporium *atrum*.

24.(once amended) The method of Claim 21 wherein the **[phenol oxidizing]** **oxidoreductase** sequence comprises the amino acid sequence as disclosed in **[SEQ ID NO:4,]** SEQ ID NO:7 or SEQ ID NO:9.

25.(Once amended) The method of Claim 21 wherein said polynucleotide comprises the sequence as shown in **[SEQ ID NO:3,]** SEQ ID NO:6, or SEQ ID NO:8.

26.(Once amended) The method of Claim 21 wherein said host cell includes filamentous fungus, yeast **[and]** **or** bacteria.

27.(Once amended) The method of Claim 26 wherein said yeast **[includes]** **is selected from the group consisting of** Saccharomyces, Pichia, Schizosaccharomyces, Hansenula, Kluyveromyces, and Yarrowia species.

28. (Once amended) The method of Claim 26 wherein said filamentous fungus **[includes] is selected from the group consisting of** Aspergillus species, Trichoderma species and Mucor species.

29.(Once amended) A method for producing a host cell comprising **[a phenol oxidizing] an oxidoreductase** enzyme comprising the steps of:

a) obtaining a polynucleotide **[capable of hybridizing] that hybridizes** to the nucleic acid having the sequence as shown in SEQ ID NO:1, or a fragment thereof, under conditions of **[high to]** intermediate stringency **which includes hybridization at about 37°C in buffer including 25% formamide and washing at about 50°C;**

b) introducing said polynucleotide into said host cell; and

c) growing said host cell under conditions suitable for the production of said **[phenol oxidizing] oxidoreductase** enzyme.

30.(Once amended) The method of Claim 29 wherein said host cell includes filamentous fungus, yeast **[and] or** bacteria.

31.(Once amended) The method of Claim 30 wherein said filamentous fungus **[includes] is selected from the group consisting of** Aspergillus species, Trichoderma species and Mucor species.

34.(Once amended) The method of Claim 33 wherein said polynucleotide comprises the nucleic acid sequence as shown in **[SEQ ID NO:3,]** SEQ ID NO:6 or SEQ ID NO:8.

STATUS OF PENDING CLAIMS

1.(Once amended) An isolated oxidoreductase enzyme encoded by a nucleic acid capable of hybridizing to the nucleic acid having the sequence as shown in SEQ ID NO:1 or a fragment thereof, under conditions of high stringency which includes hybridization at about 37°C in buffer including 50% formamide and washing at about 65°C, said isolated oxidoreductase having at least 80% sequence identity to the amino acid sequence disclosed in SEQ ID NO: 2, wherein said isolated oxidoreductase enzyme is obtainable from a source other than *Stachybotrys chartarum*.

2.(Once amended) The oxidoreductase enzyme of Claim 1 having at least 60% identity to the oxidoreductase enzyme having the amino acid sequence as disclosed in SEQ ID NO:2.

3.(Once amended) The oxidoreductase enzyme of Claim 1, wherein said enzyme is obtained from a bacteria, yeast or non-*Stachybotrys* fungus.

4.(Once amended) The oxidoreductase enzyme of Claim 3 wherein said fungus is selected from the group consisting of Myrothecium species, Curvularia species, Chaetomium species, Bipolaris species, Humicola species, Pleurotus species, Trichoderma species, Mycellophthora species and Amerosporium species.

5.(Once amended) The oxidoreductase enzyme of Claim 4 wherein the fungus are selected from the group consisting of Myrothecium verrucaria, Curvalaria *pallescens*, Chaetomium sp, Bipolaris *spicifera*, Humicola insolens, Pleurotus abalonus, Trichoderma reesei, Mycellophthora thermophila and Amerosporium *atrum*.

6.(Once amended) The oxidoreductase enzyme of Claim 4 wherein said fungus is a Bipolaris species, a Curvularia species or a Amerosporium species.

7.(Once amended) The oxidoreductase enzyme of Claim 6 wherein said fungus is Bipolaris *spicifera*, Curvularia *pallescens* or Amerosporium *atrum*.

8.(Once Amended) An isolated oxidoreductase enzyme comprising an amino acid sequence having at least 95% identity to the amino acid sequence disclosed in SEQ ID NO:4, SEQ ID NO:7 or SEQ ID NO:9.

9.(Once amended) An isolated polynucleotide encoding the amino acid comprising the sequence as shown in SEQ ID NO:7 or SEQ ID NO:9.

10.(Reitered) The isolated polynucleotide of Claim 9 having at least 60% identity to the nucleic acid sequence disclosed in SEQ ID NO:1 or SEQ ID NO:3.

11.(Once amended) The isolated polynucleotide of Claim 9 comprising the nucleic acid sequence as disclosed in SEQ ID NO:6 or SEQ ID NO:8.

12.(Once amended) An isolated polynucleotide encoding an oxidoreductase enzyme that hybridizes to the sequence shown in SEQ ID NO: 3, SEQ ID NO:6 or SEQ ID NO:8 or a fragment thereof, under conditions of intermediate stringency which includes hybridization at about 37°C in buffer including 25% formamide and washing at about 50°C.

13.(Reitered) An expression vector comprising the polynucleotide of Claim 10.

14.(Reitered) A host cell comprising the expression vector of Claim 13.

15.(Reitered) The host cell of Claim 14 that is a filamentous fungus.

16.(Once amended) The host cell of Claim 15 wherein said filamentous fungus is selected form the group consisting of Aspergillus species, Trichoderma species and Mucor species.

17.(Reitered) The host cell of Claim 14 that is a yeast.

18.(Once amended) The host cell of Claim 17 wherein said yeast is selected from the group consisting of Saccharomyces, Pichia, Schizosaccharomyces, Hansenula, Kluyveromyces, and Yarrowia species.

19.(Reitered) The host cell of Claim 14 wherein said host is a bacterium.

20.(Once amended) The host cell of Claim 19 wherein said bacterium is selected from the group consisting of Bacillus and Escherichia species.

21.(Once amended) A method for producing an oxidoreductase enzyme in a host cell comprising the steps of:

a) obtaining a host cell comprising a polynucleotide that hybridizes to the nucleic acid shown in SEQ ID NO:1, or a fragment thereof, under conditions of intermediate stringency which includes hybridization at about 37° C in buffer including 25% formamide and washing at about 50°C ; and

b) growing said host cell under conditions suitable for the production of said oxidoreductase enzyme.

22.(Reitered) The method of Claim 21 wherein said phenol oxidizing enzyme is obtainable from Myrothecium species, Curvalaria species, Chaetomium species, Bipolaris species, Humicola species, Pleurotus species, Trichoderma species, Mycellophthora species or Amerosporium species.

23.(Once amended) The method of Claim 22 wherein the fungus is selected from the group consisting of Myrothecium verrucaria, Curvalaria *pallescens*, Chaetomium sp, Bipolaris *spicifera*, Humicola insolens, Pleurotus abalonus, Trichoderma reesei, Mycellophthora thermophila or Amerosporium *atrum*.

24.(Once amended) The method of Claim 21 wherein the oxidoreductase sequence comprises the amino acid sequence as disclosed in SEQ ID NO:7 or SEQ ID NO:9.

25.(Once amended) The method of Claim 21 wherein said polynucleotide comprises the sequence as shown in SEQ ID NO:6 or SEQ ID NO:8.

26.(Once amended) The method of Claim 21 wherein said host cell includes filamentous fungus, yeast or bacteria.

27.(Once amended) The method of Claim 26 wherein said yeast is selected from the group consisting of Saccharomyces, Pichia, Schizosaccharomyces, Hansenula, Kluyveromyces, and Yarrowia species.

28. (Once amended) The method of Claim 26 wherein said filamentous fungus is selected from the group consisting of Aspergillus species, Trichoderma species and Mucor species.

29.(Once amended) A method for producing a host cell comprising an oxidoreductase enzyme comprising the steps of:

a) obtaining a polynucleotide that hybridizes to the nucleic acid having the sequence as shown in SEQ ID NO:1, or a fragment thereof, under conditions of intermediate stringency which includes hybridization at about 37°C in buffer including 25% formamide and washing at about 50°C;

b) introducing said polynucleotide into said host cell; and

c) growing said host cell under conditions suitable for the production of said oxidoreductase enzyme.

30.(Once amended) The method of Claim 29 wherein said host cell includes filamentous fungus, yeast or bacteria.

31.(Once amended) The method of Claim 30 wherein said filamentous fungus is selected from the group consisting of Aspergillus species, Trichoderma species and Mucor species.

32.(Reitered) The method of Claim 31 wherein said Aspergillus species is Aspergillus *niger var. awamori.*

33.(Reitered) The method of Claim 29 wherein said polynucleotide has at least 60% identity to the nucleic acid shown in SEQ ID NO:1 or SEQ ID NO:3.

34.(Once amended) The method of Claim 33 wherein said polynucleotide comprises the nucleic acid sequence as shown in SEQ ID NO:6 or SEQ ID NO:8.

35. The oxidoreductase enzyme of Claim 2, wherein said enzyme has at least 90% identity to the amino acid sequence of SEQ ID NO: 2.

36. The isolated oxidoreductase of Claim 8, wherein said oxidoreductase enzyme has an amino acid sequence having at least 95% identity to the sequence disclosed in SEQ ID NO: 4

37. The isolated oxidoreductase enzyme of Claim 8, wherein said oxidoreductase enzyme has an amino acid sequence having at least 95% identity to the sequence disclosed in SEQ ID NO: 7

38. The isolated oxidoreductase enzyme of Claim 8, wherein said oxidoreductase enzyme has an amino acid sequence having at least 95% identity to the sequence disclosed in SEQ ID NO: 9.

39. An isolated polynucleotide encoding the oxidoreductase enzyme of Claim 8.

40. An expression vector comprising the polynucleotide of Claim 9.

41. A host cell comprising the expression vector of Claim 40.

42. The host cell of Claim 41 that is a filamentous fungus.

43. The host cell of Claim 42 wherein said filamentous fungus is selected form the group consisting of Aspergillus species, Trichoderma species and Mucor species.

44. The host cell of Claim 41 that is a yeast.

45. The host cell of Claim 44 wherein said yeast is selected from the group consisting of Saccharomyces, Pichia, Schizosaccharomyces, Hansenula, Kluyveromyces, and Yarrowia species.

46. The host cell of Claim 41 wherein said host is a bacterium.

47. The host cell of Claim 46 wherein said bacterium is a *Bacillus* species.

48. The method for producing an oxidoreductase enzyme according to Claim 21 further comprising recovering said oxidoreductase from step b).

49. A method for producing an oxidoreductase enzyme in a host cell comprising the steps of:

a) obtaining a host cell comprising a polynucleotide that hybridizes to the nucleic acid shown in SEQ ID NO:1, or a fragment thereof, under conditions of high stringency which includes hybridization at about 37 °C in buffer including 50% formamide and washing at about 65°C, and

b) growing said host cell under conditions suitable for the production of said oxidoreductase enzyme.

50. The method of Claim 49 further comprising recovering said oxidoreductase from step b).